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Involvement of RNA2-Encoded Proteins in the Specific Transmission of *Grapevine Fanleaf Virus* by Its Nematode Vector *Xiphinema index*

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The nepovirus *Grapevine fanleaf virus* (GFLV) is specifically transmitted by the nematode *Xiphinema index.* To identify the RNA2-encoded proteins involved in *X. index*-mediated spread of GFLV, chimeric RNA2 constructs were engineered by replacing the 2A, $2B^{MP}$, and/or $2C^{CP}$ sequences of GFLV with their counterparts in *Arabis mosaic virus* (ArMV), a closely related nepovirus which is transmitted by *Xiphinema diversicaudatum* but not by *X. index.* Among the recombinant viruses obtained from transcripts of GFLV RNA1 and chimeric RNA2, only those which contained the $2C^{CP}$ gene (504 aa) and $2B^{MP}$ contiguous 9 C-terminal residues of GFLV were transmitted by *X. index* as efficiently as natural and synthetic wild-type GFLV, regardless of the origin of the 2A and $2B^{MP}$ genes. As expected, ArMV was not transmitted probably because it is not retained by *X. index.* These results indicate that the determinants responsible for the specific spread of GFLV by *X. index* are located within the 513 C-terminal residues of the polyprotein encoded by RNA2.

INTRODUCTION

Grapevine fanleaf virus (GFLV) is responsible for a progressive degeneration of grapevines that occurs in vineyards worldwide (Pearson and Goheen, 1991). It causes serious economic losses by substantially reducing yield and affecting fruit quality. GFLV belongs to the plant virus family *Comoviridae* and to the genus *Nepovirus,* which is characterized by isometric particles of ca. 30 nm in diameter, a bipartite RNA genome, and a nematode-vectored transmission, among other characteristics (Mayo and Robinson, 1996).

The genome of GFLV is composed of two single-stranded positive-sense RNAs, called RNA1 and RNA2, which carry a small covalently linked viral protein (VPg) at their 5′ extremities and a poly(A) stretch at their 3′ ends (Fig. 1A) (Pinck *et al.,* 1988). GFLV strain F13 (GFLV-F13) includes a satellite RNA, designated RNA3, which encodes a 37-kDa peptide (Fuchs *et al.,* 1989). GFLV genomic RNAs code for polyproteins from which functional proteins are generated by proteolytic processing at defined dipeptide cleavage sites (Fig. 1A). RNA1 codes for the proteins implicated in RNA replication and for the viral proteinase (Margis *et al.,* 1991; Ritzenthaler *et al.,* 1991). RNA2 encodes three final cleavage products (Margis *et al.,* 1993): the N-proximal 2A protein (28 kDa), which is required for RNA2 replication (Gaire *et al.,* 1999); the central $2B^{MP}$ protein (38 kDa), which is needed for cell-to-cell movement and is the constituent protein of tubules observed in plasmodesmata (Ritzenthaler *et al.,* 1995b); and the C-proximal $2C^{CP}$ capsid protein (56 kDa) (Serghini *et al.,* 1990), which is also necessary for virus spread. Full-length cDNA clones of GFLV RNA1 and RNA2 have been constructed for the synthesis of infectious transcripts (Viry *et al.,* 1993).

GFLV is naturally transmitted from grapevine to grapevine by the ectoparasitic nematode *Xiphinema index* (Martelli and Taylor, 1990). The feeding of *X. index* occurs at actively growing root tips and induces the formation of galls which contain enlarged multinucleate cells with dense cytoplasm (Brown *et al.,* 1995; Wyss, 2000). The transmission process is characterized by a high degree of specificity between GFLV and *X. index.* This specificity is determined by the nematode's ability to ingest GFLV particles from a virus source plant, retain them at specific retention sites within its feeding apparatus, and subsequently infect a recipient plant upon release of virus particles from the retention sites.

Limited information is available on the mechanisms of the transmission process of nepoviruses, including GFLV (Brown *et al.,* 1995; Mayo *et al.,* 1994; Mayo and Robinson, 1996; Wyss, 2000). Experiments with pseudorecombinants of two nepoviruses, Raspberry ringspot virus (RpRSV) and *Tomato black ring virus* (TBRV), revealed that transmissibility segregates with RNA2 (Harrison and Murant, 1977; Harrison *et al.,* 1974a). Studies on RpRSV transmission were based on the potential of its nematode vector, *Longidorus elongatus,* to transmit strains S more efficiently than strains E. Harrison *et al.* (1974a) showed that a hybrid RpRSV containing RNA2 from strain

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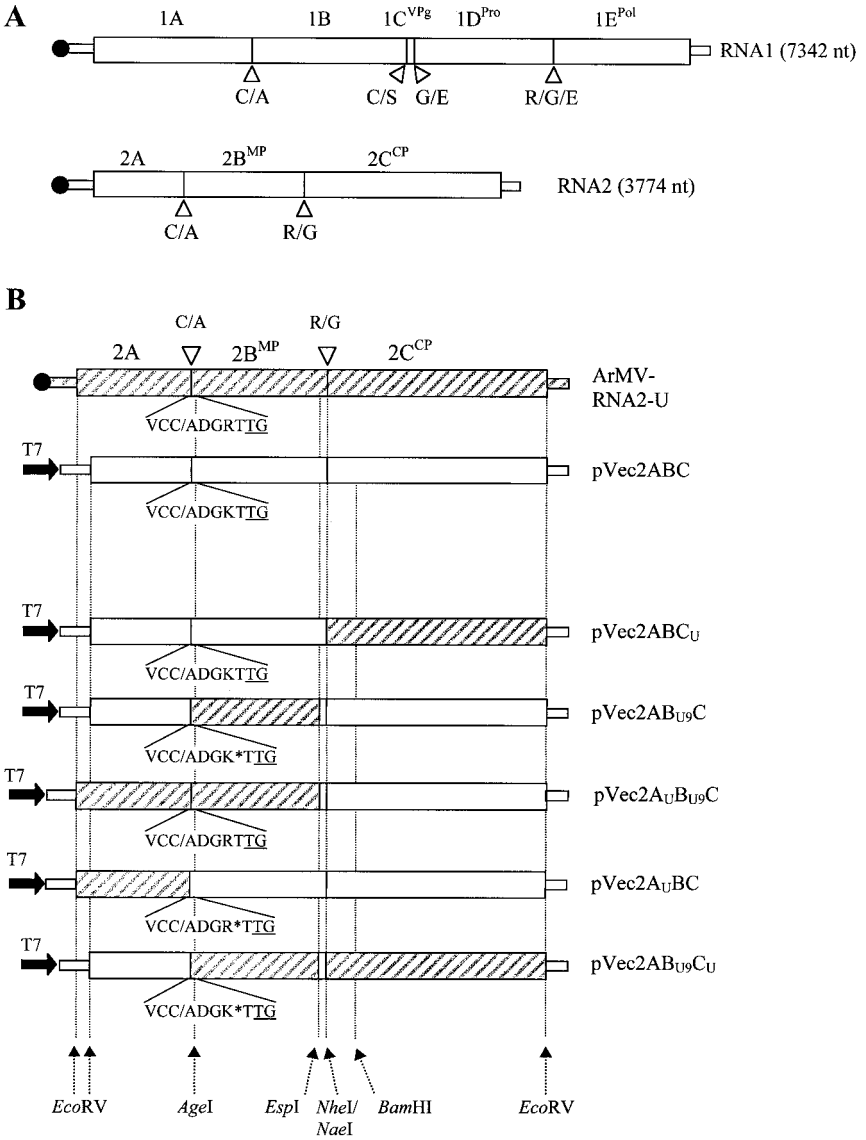

FIG. 1. (A) Genetic organization of GFLV-F13 RNA1 and RNA2. Wide white boxes represent ORFs. The 5′ and 3′ noncoding regions are denoted by narrow boxes and the VPg is represented by a black circle. Open triangles indicate the cleavage sites. The name of the processed proteins is given on top of the boxes: VPg, genome-linked protein; Pro, proteinase; Pol, polymerase; MP, movement protein; CP, coat protein. (B) Schematic representation of recombinant RNA2 used in this study. Open boxes indicate GFLV sequences and hatched boxes indicate ArMV RNA2-U sequences. The C/A and R/G cleavage sites are marked by an inverted open triangle. The positions of the restriction sites (*Eco*RV, *Age*I, *Esp*I, *Nhe*I/*Nae*I, and *Bam*HI) used for the construction of the chimera are indicated by thin arrows. The T7 promoter is represented by a thick black arrow. The context of the C/A cleavage site is shown below the construct. Underlined amino acids correspond to the position of the *Age*I restriction site. Residue K* (originating from the GFLV $2B^{MP}$ gene) in constructs pVec2AB_{U9}C and pVec2A$B_{U9}C_U$ and residue R* (originating from ArMV RNA2-U $2B^{MP}$ gene) in construct pVec2A_UBC are the only modifications resulting from the cloning strategy. The ArMV RNA2-U original R is restored in construct pVec2A_UB_{U9}C.

S exhibits a substantially higher transmission frequency than a hybrid with RNA2 from strain E. Also, transmissibility by *L. elongatus* of pseudorecombinants containing RNA1 and RNA2 from two distinct serotypes of TBRV was correlated with the serological characteristics of the virus particles (Harrison and Murant, 1977).

Despite many hypotheses on the functions of nepoviral RNA2-encoded proteins in nematode transmission (see for review Mayo *et al.,* 1994), there is no direct evidence so far to impute vector specificity to any of the three proteins: 2A, $2B^{MP}$, and/or $2C^{CP}$.

In this study, we investigated the involvement of RNA2-encoded proteins in the *X. index*-vectored transmission of GFLV using reverse genetics and the nepovirus *Arabis mosaic virus* (ArMV). ArMV and GFLV have a similar genome organization and expression and are serologically distinctly related (Martelli and Taylor, 1990). Noteworthy, ArMV strain S contains a mixture of two distinct

RNA2s, called RNA2-U and RNA2-L, which slightly differ in size and sequence identity with the GFLV-encoded polyprotein P2 (Loudes *et al.,* 1995). Despite high sequence identities among the GFLV and ArMV RNA2-encoded proteins, i.e., 56–62, 86–88, and 69% between the 2A, $2B^{MP}$, and $2C^{CP}$ domains, respectively, GFLV is transmitted by *X. index* and ArMV by *Xiphinema diversicaudatum* (for a review see Brown *et al.,* 1995). To study the molecular basis of the vector specificity in GFLV, our experimental approach was to: (1) Engineer chimeric RNA2 constructs by replacing the GFLV 2A, $2B^{MP}$, and $2C^{CP}$ genes with their counterparts in ArMV, (2) Develop transcripts of chimeric RNA2 constructs, (3) Establish systemic infection of host plants with transcripts of GFLV RNA1 and chimeric RNA2 constructs, and (4) Evaluate the transmissibility of the recombinant viruses by *X. index.* We show that replacing the GFLV 2A and 2B genes with those of ArMV did not greatly alter the transmission of recombinant viruses. This is the first molecular study on the specificity of vector-mediated transmission of a nepovirus.

RESULTS

Characteristics of RNA2 chimera

To identify the RNA2-encoded proteins involved in the specific transmission of GFLV by its nematode vector *X. index,* chimeric RNA2 constructs were engineered by replacing the 2A, $2B^{MP}$, and/or $2C^{CP}$ sequences of GFLV with their counterparts in ArMV. All chimeric RNA2 constructs were engineered from pVec2ABC (Fig. 1B), a pUC-based cloning vector which contains the full-length GFLV RNA2 sequence (Belin *et al.,* 1999). Since previous experiments indicated that the two RNA2 constituents of ArMV-S, RNA2-U and RNA2-L (Loudes *et al.,* 1995), are biologically indistinguishable (Belin *et al.,* 1999), only ArMV RNA2-U sequences were used in this study.

Five chimeric GFLV/ArMV RNA2 constructs were engineered. In the first construct, the $2C^{CP}$ gene of ArMV RNA2-U was inserted into pVec2ABC to yield $pVec2ABC_U$ (U as subscript indicates the ArMV RNA2-U origin of the gene) (Fig. 1B) (Belin *et al.,* 1999). In the second construct, the $2B^{MP}$ coding sequence was derived from ArMV RNA2-U in $pVec2AB_{U9}C$ (Belin *et al.,* 1999), except for residue +4 downstream of the C/A cleavage site (K* in Fig. 1B) and for the 9 C-terminal residues which are of GFLV origin (9 as subscript indicates the GFLV origin of the 9 C-terminal amino acids of protein $2B^{MP}$). In the third construct, the 2A coding sequence of GFLV was replaced by its ArMV RNA2-U counterpart in $pVec2AB_{U9}C$ to yield $pVec2A_UB_{U9}C$ (Fig. 1B). This exchange restored the original Arg at position +4 of protein $2B_U$. In the fourth construct, the $2B^{MP}$ coding sequence of GFLV was cloned into $pVec2A_UB_{U9}C$ to yield $pVec2A_UBC$. This insertion substituted Lys by Arg at position +4 downstream of the C/A cleavage site (R* in Fig. 1B). In the fifth construct, the $2C^{CP}$ coding sequence of GFLV was exchanged by the ArMV RNA2-U counterpart in $pVec2AB_{U9}C$ to yield $pVec2AB_{U9}C_U$ (Fig. 1B) (Belin *et al.,* 1999).

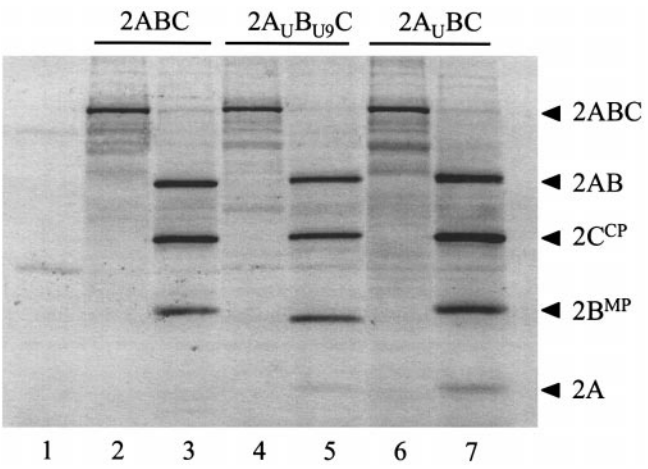

FIG. 2. *In vitro* translation and processing of recombinant transcripts $Tr2A_UB_{U9}C$ (lanes 4 and 5) and $Tr2A_UBC$ (lanes 6 and 7) and control transcript Tr2ABC (lanes 2 and 3). Transcripts were incubated in rabbit reticulocyte lysate with [^{35}S]methionine in the presence (lanes 3, 5, and 7) or in the absence (lanes 2, 4, and 6) of the transcript deriving from plasmid pET-Pro, which encodes the GFLV proteinase $1D^{Pro}$. Lane 1, rabbit reticulocyte lysate with [^{35}S]methionine in the absence of transcript. Proteins were analyzed on a 10% SDS–polyacrylamide gel. The positions of the maturation intermediates and final cleavage products are shown on the right.

Proteolytic processing of chimeric RNA2-encoded polyproteins

The processing of polyproteins P2 encoded by transcripts of pVec2ABC and chimeric $pVec2A_UBC$, $pVec2AB_{U9}C$, $pVec2A_UB_{U9}C$, $pVec2ABC_U$, and $pVec2AB_{U9}C_U$ was analyzed to ensure that sequence modifications did not induce a dramatic decrease in maturation efficiency by the GFLV RNA1-encoded proteinase.

Belin *et al.* (1999) previously showed that polyproteins P2ABC, $P2AB_{U9}C$, $P2ABC_U$, and $P2AB_{U9}C_U$ are completely processed in *Chenopodium quinoa* protoplasts transfected with transcripts corresponding to RNA1 (Tr1) and chimeric RNA2 (Tr2). No maturation intermediates were obtained, indicating a complete cleavage at the C/A site between the 2A and the $2B^{MP}$ proteins, and at the R/G site between the $2B^{MP}$ and the $2C^{CP}$ proteins. These results demonstrated that transcripts corresponding to 2ABC, $2AB_{U9}C$, $2ABC_U$, and $2AB_{U9}C_U$ are translationally functional.

For the polyproteins P2 encoded by transcripts of $pVec2A_UBC$ and $pVecA_UB_{U9}C$, *in vitro* translation and processing experiments showed that the relative ratio between the polypeptides 2ABC, the fully processed $2B^{MP}$ and $2C^{CP}$ proteins, or the maturation intermediates 2AB was similar for 2ABC (Fig. 2, lanes 2 and 3), $2A_UB_{U9}C$ (Fig. 2, lanes 4 and 5), and $2A_UBC$ (Fig. 2, lanes 6 and 7).

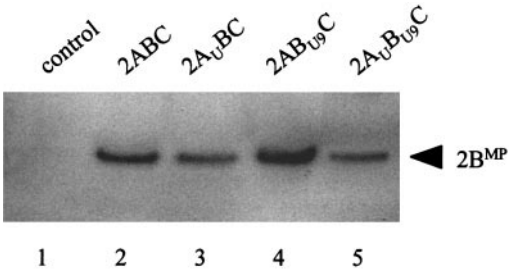

FIG. 3. Detection of protein 2B^{MP} by Western blot analysis in *C. quinoa* plants mechanically inoculated with transcripts Tr1 and Tr2ABC (lane 2), Tr1 and Tr2A$_U$BC (lane 3), Tr1 and Tr2AB$_{U9}$C (lane 4), and Tr1 and Tr2A$_U$B$_{U9}$C (lane 5). Lane 1, healthy plant. At 10 days postinoculation, noninoculated apical leaves were ground in loading buffer, and extracted total plant proteins were separated by electrophoresis on a 15% SDS–polyacrylamide gel and probed with antibodies raised against the GFLV 2B^{MP} protein after transfer to an Immobilon membrane. The position of the 2B^{MP} protein is indicated on the right.

These results indicate that protein 2A from ArMV does not affect the *in vitro* maturation of polyprotein P2.

Therefore, the exchange of the 2A, 2B^{MP}, and/or 2C^{CP} genes for the five chimeric RNA2 constructs used in this study has no effect on the polyprotein P2 proteolytic processing by the GFLV RNA1-encoded proteinase.

Systemic infection of GFLV RNA1 and recombinant RNA2 *in planta*

To investigate the biological properties of the recombinant constructs and the occurrence of systemic infection *in planta, C. quinoa* plants were mechanically inoculated with transcripts of GFLV RNA1 and chimeric RNA2.

Belin *et al.* (1999) previously showed that synthetic wild-type GFLV corresponding to Tr1 and Tr2ABC and the recombinant virus corresponding to Tr1 and Tr2AB$_{U9}$C systemically infect *C. quinoa* plants. In contrast, the recombinant viruses corresponding to Tr1 and Tr2ABC$_U$ and to Tr1 and Tr2AB$_{U9}$C$_U$ do not infect *C. quinoa,* despite the occurrence of RNA replication, particle assembly, and tubule formation (Belin *et al.,* 1999).

In this study, we found that the recombinant viruses corresponding to Tr1 and Tr2A$_U$BC and to Tr1 and Tr2A$_U$B$_{U9}$C systemically infect *C. quinoa* with mosaic symptoms developing in noninoculated apical leaves. Furthermore, protein 2B^{MP} accumulated to approximately the same level in plants infected with these recombinant viruses as in plants inoculated with Tr1 and Tr2ABC, as determined by Western blotting (Fig. 3). These results suggest that replication, translation, *in vivo* processing, and systemic movement of chimeric viruses 2A$_U$BC and 2A$_U$B$_{U9}$C are not impaired by the exchange of the 2A and/or 2B^{MP} proteins.

Natural wild-type viruses GFLV-F13 and ArMV-S, synthetic wild-type virus 2ABC, and recombinant viruses 2A$_U$BC, 2AB$_{U9}$C, and 2A$_U$B$_{U9}$C were further propagated in *C. quinoa.* Subsequently, infected *C. quinoa* were used to inoculate grapevine plants by heterologous grafting *in vitro.*

Some of the viruses were also purified from infected *C. quinoa* and used to mechanically inoculate petunia, a herbaceous plant proposed for *X. index*-mediated virus transmission experiments (Coiro and Serino, 1991) which allows an asymptomatic multiplication of GFLV (this study) and ArMV (Brown *et al.,* 1995).

Infected grapevines and petunia were used as virus source plants in nematode transmission experiments.

Transmissibility of recombinant viruses by *X. index*

The transmissibility of natural and synthetic wild-type viruses and of recombinant viruses by *X. index* was examined in the greenhouse using aviruliferous nematodes. Nematode vectors were from figs, a host plant on which GFLV does not multiply. The presence of the viruses was verified in roots of inoculated petunia and grapevine plants by enzyme-linked immunosorbent assay (ELISA) prior to nematode transmission tests. Aviruliferous nematodes were allowed to feed for 6 weeks on roots of infected petunia or grapevine plants. After completion of the virus acquisition step, virus source plants were uprooted and replaced by healthy grapevines. Nematodes were then allowed to feed for 6 additional weeks.

The roots of source and recipient plants were examined for gall formation as indicative of nematode feeding. Numerous galls were observed on roots of source and recipient grapevines but not on petunia (data not shown).

Recombinant viruses 2A$_U$BC, 2AB$_{U9}$C, and 2A$_U$B$_{U9}$C were detected by ELISA in rootlets of 17 of 17 (100%), 27 of 31 (87%), and 8 of 9 (89%) recipient grapevine plants, respectively, 6 weeks posttransmission (Table 1). The spread of these three recombinant viruses was confirmed by ELISA 5 months later in major roots of 100 (12/12), 83 (15/18), and 86% (6/7) of the recipient plants, respectively, and 6–8 months later in leaves of 25 (1/4), 81 (13/16), and 100% (3/3) of the recipient plants, respectively. The recombinant viruses 2A$_U$BC, 2AB$_{U9}$C, and 2A$_U$B$_{U9}$C were also found in newly developed leaves of 2 of 2, 16 of 27, and 3 of 4 plants after a 6-week dormancy period, respectively. These results indicate that *X. index*-vectored transmission of the recombinant viruses 2A$_U$BC, 2AB$_{U9}$C, and 2A$_U$B$_{U9}$C readily occurred and resulted in systemic infection of the recipient plants.

As expected, natural wild-type GFLV-F13 and synthetic wild-type 2ABC were detected in the rootlets of 100 (11/11) and 95% (18/19) of the recipient plants, respectively (Table 1). Further, the major roots of all recipient plants (30/30) and the aerial parts of most of the recipient plants (9/10) were infected 5 months after completion of the transmission test. Also, the virus was present in the leaves of all the plants tested after a dormancy period

TABLE 1

Transmissibility of Natural Wild-Type GFLV-F13 and ArMV-S, Synthetic GFLV Wild-Type 2ABC, and Recombinant Viruses Derived from Transcripts of GFLV RNA1 and Chimeric RNA2 by *Xiphinema index*

Inoculum	Virus source plant	No.		Transmission[a]	%
GFLV-F13	Petunia	5		5/5	
	Grapevine	6		6/6	
			Total	11/11	100
2ABC	Petunia	10		10/10	
	Grapevine	9		8/9	
			Total	18/19	95
$2A_UBC$	Petunia	nd		nd	
	Grapevine	17		17/17	
			Total	17/17	100
$2AB_{U9}C$	Petunia	8		8/8	
	Grapevine	23		19/23	
			Total	27/31	87
$2A_UB_{U9}C$	Petunia	nd		nd	
	Grapevine	9		8/9	
			Total	8/9	89
ArMV-S	Petunia	8		0/8	
	Grapevine	8		0/8	
			Total	0/16	0
Mock	Petunia	3		0/3	
	Grapevine	3		0/3	
			Total	0/6	0

[a] Data correspond to the number of recipient plants that reacted positively for GFLV in ELISA over the total number of recipient plants tested. Positive recipient plants had $OD_{405\,nm}$ values of 0.22–2.52 compared to 0.01–0.03 for healthy control plants after 30 min of substrate hydrolysis. Each recipient grapevine plant was maintained for 6 weeks in contact with *X. index* which previously fed on a single virus source plant. See Materials and Methods for a description of the recombinant viruses. No., number of test plants; nd, not determined.

(4/4). In contrast, ArMV-S was not detected in roots of any of the 16 recipient plants, confirming that ArMV is not transmissible by *X. index* (Table 1). Similarly, no transmission was obtained when mock-inoculated plants were used as virus source plants. These results indicate that the transmission of the recombinant viruses $2A_UBC$, $2A_UB_{U9}C$, and $2AB_{U9}C$ by *X. index* is equally as efficient as that of natural wild-type GFLV-F13 and synthetic wild-type 2ABC, suggesting that proteins $2A_U$ and $2B_{U9}$, either independently or in association, do not affect the specificity of GFLV transmission by *X. index*. In addition, transmission was equally as efficient with petunia and grapevine as virus source plants since 100 (23/23) and 91% (58/64) of the recipient grapevine plants became infected with transmissible viruses (Table 1).

Characterization of recombinant virus progeny

The progeny of recombinant viruses $2A_UBC$, $2AB_{U9}C$, and $2A_UB_{U9}C$ were characterized by sequencing to determine their stability because mutations can occur or be selected during virus replication, systemic host infection or change in host plant, and vector transmission (Ayllon *et al.,* 1999; Roossinck, 1997). The RNA2 coding region of recombinant viruses was completely sequenced from six to seven cDNA fragments which were amplified by polymerase chain reaction (PCR) from rootlets of three to five recipient plants (Fig. 4 and Table 2).

The sequence information clearly showed that the progeny of recombinant viruses $2A_UBC$, $2AB_{U9}C$, and $2A_UB_{U9}C$ were stable because only very limited nucleotide changes were observed for each chimeric RNA2. Indeed, only 1–2 of the 3310–3380 (0.03–0.06%) nucleo-

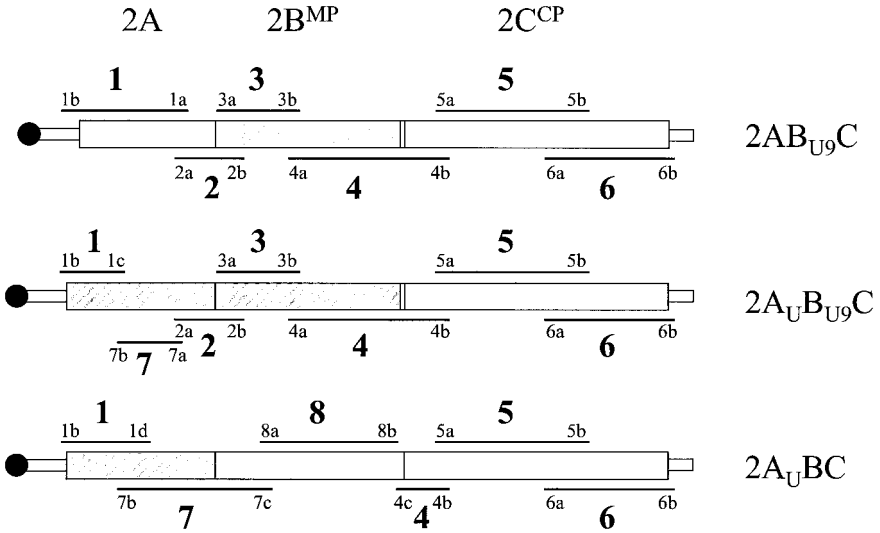

FIG. 4. Strategy to sequence full-length RNA2 of the progeny of recombinant viruses $2A_UBC$, $2AB_{U9}C$, and $2A_UB_{U9}C$ after nematode transmission. The size and position of the IC-RT-PCR fragments (1–8) used for sequencing are shown to scale. The name of the primers used for amplification is indicated at the extremities of the corresponding fragments. A description of the eight fragments and 20 primers is given in Table 2. Open and hatched boxes indicate GFLV and ArMV sequences, respectively. The 5′ and 3′ noncoding regions are denoted by narrow boxes and the VPg is represented by a black circle.

TABLE 2

Oligonucleotides and PCR Fragments Used for Sequencing the Progeny of Recombinant Viruses $2A_UBC$, $2AB_{U9}C$, and $2A_UB_{U9}C$

PCR fragment	Primer				PCR-fragment length		
	Name	GFLV position	ArMV position	Sequence	$2AB_{U9}C$	$2A_UB_{U9}C$	$2A_UBC$
1	1a	867–850 (−)	n/a	5′ GAGGATTTTGGATTGGGGG 3′			
	1b	135–158 (+)	n/a	5′ CTTTTTTGTTCTTTTATTTTGCGC 3′	732 bp		
	1c	n/a	599–582 (−)	5′ CCCGACAGATCGTTGCAC 3′		436 bp	
	1d	n/a	742–758 (−)	5′ TGACTCTTCCCGTTCCC 3′			651 bp
2	2a	784–803 (+)	872–891 (+)	5′ GTGGTATGACACTAGTGATG 3′			
	2b	n/a	1273–1254 (−)	5′ TCAGTTTGAGCAGCAAGACC 3′	392 bp	401 bp	—
3	3a	998–1005 (+)	1101–1122 (+)	5′ TGCTGTGCTGATGGTAGGACTACCGGTG 3′			
	3b	n/a	1579–1560 (−)	5′ TTGCCTGGCATTCCAAAAGG 3′	484 bp	484 bp	—
4	4a	n/a	1508–1525 (+)	5′ TGACAGGGGTATGTCAGC 3′			
	4b	2316–2297 (−)	n/a	5′ AAGGTGTATACAGGATCCGC 3′	880 bp	880 bp	
	4c	2015–2035 (+)	n/a	5′ GAGGCTGAGCCCAGACTGAGC 3′			301 bp
5	5a	2234–2254 (+)	n/a	5′ GGATTGACATGGGTGATGAGC 3′			
	5b	3103–3083 (−)	n/a	5′ ATCCACCCATACGAAATAGTC 3′	849 bp	849 bp	849 bp
6	6a	2846–2867 (+)	n/a	5′ AAGTATCCCGGGGTGTATGTGG 3′			
	6b	3590–3570 (−)	n/a	5′ ACACTTGGGTCTTTTAAAGTC 3′	724 bp	724 bp	724 bp
7	7a	n/a	920–903 (−)	5′ ACCACCAGGATGAGTGCG 3′			
	7b	n/a	553–568 (+)	5′ CCGAGCCGGTTAAAGC 3′	—	367 bp	
	7c	1315–1335 (−)	n/a	5′ TTGAGCCTAAAATCTAGCGTG 3′			878 bp
8	8a	1267–1288 (+)	n/a	5′ TTACGCCCCTAGGGGTTTGTGG 3′			
	8b	2024–2041 (−)	n/a	5′ GGTTGAGCTCAGTCTGGG 3′	—	—	774 bp

Note. The position of the primers is numbered after Serghini *et al.* (1990) and Loudes *et al.* (1995) for GFLV RNA2 (GenBank Accession No. X16907) and ArMV RNA2-U (GenBank Accession No. X81814) genes, respectively. The corresponding positive and negative strands of RNA2 are indicated in parentheses for each primer. n/a, not applicable.

tides determined were modified compared to the reference GFLV (Serghini *et al.,* 1990) and ArMV (Loudes *et al.,* 1995) sequences.

Modifications in $2A_UB_{U9}C$ progeny affected the $2A_U$ domain but not the $2B_{U9}$ and $2C^{CP}$ sequences with nucleotides C^{596} and A^{540} changed into U^{596} and G^{540}, respectively. The former modification was silent but the latter converted amino acid M^{94} to V^{94}. To evaluate the importance of this change with regard to the transmission process, another recipient grapevine and the virus source plant corresponding to the recipient plant from which the sequenced fragment was amplified (fragment 1 in Fig. 4) were tested. Data clearly show that M^{94} was unchanged in the new recipient plant as well as in the virus source plant, suggesting that modification at position 94 is not critical for transmission.

Modifications in $2AB_{U9}C$ progeny affected only the $2B_{U9}$ sequence but not the 2A and $2C^{CP}$ domains with nucleotides A^{241} and C^{1202} changed into G^{241} and C^{1202}, respectively. The first modification was silent, whereas the second changed amino acid I^{292} to M^{292}. This mutation was not found in another recipient grapevine nor in the $2AB_{U9}C$ inoculum, suggesting that it is not critical for virus spread.

The only modification detected in $2A_UBC$ progeny affected the $2A_U$ domain with nucleotide C^{523} changed into U^{523}. Consequently, amino acid A^{88} was converted to V^{88}. This mutation was present in the corresponding source grapevine but was not found in another recipient plant, suggesting that it is not required for virus transmission.

Taken together, the sequence information showed that recombinant viruses $2A_UBC$, $2AB_{U9}C$, and $2A_UB_{U9}C$ are transmitted by *X. index* with only very minor and nonessential modifications.

Retention of GFLV and ArMV by *X. index*

Nematodes can passively ingest nepoviruses during feeding (Brown *et al.,* 1995). Therefore, we wanted to know if the lack of transmissibility of ArMV by *X. index* is due to a lack of retention of virus particles at specific sites on the walls of the food canal or to an ineffective release of particles that could be ingested and retained, as described for RpRSV-S in the nematode *Longidorus macrosoma* (reviewed in Brown *et al.,* 1995; Mayo *et al.,* 1994). To discriminate between these two possibilities, the presence of ArMV in *X. index* was investigated by immunocapture–reverse transcription–polymerase chain reaction (IC-RT-PCR) 6 weeks after nematode feeding on ArMV-infected grapevines (Fig. 5).

Although ArMV was readily detected in grapevine source plants, no ArMV-specific DNA product was obtained from as many as 100 nematodes (Fig. 5, lanes 1 and 2). In contrast, a GFLV-specific PCR fragment was amplified from only 30 nematodes (Fig. 5, lane 3).

These results clearly indicate that ArMV is not retained

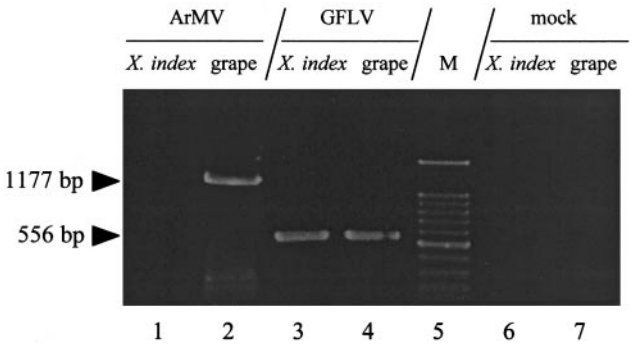

FIG. 5. Detection of GFLV and ArMV by IC-RT-PCR in *Xiphinema index* and grapevines. The nematodes analyzed were exposed to grapevines infected with ArMV (lane 1) or GFLV (lane 3) or to healthy grapevines (lane 6). Grapevines were healthy (lane 7) or infected with ArMV (lane 2) and GFLV (lane 4). Lane 5, molecular weight markers. One hundred nematodes were used for healthy and ArMV-infected plants, whereas 30 nematodes were used for GFLV-infected plants. DNA products were analyzed by electrophoresis on a 1% agarose gel. The positions of the 1177-bp ArMV-specific and 556-bp GFLV-specific fragments are shown on the left.

by *X. index* or is eventually retained below the detection threshold of IC-RT-PCR. Thus, the lack of ArMV transmissibility by *X. index* is likely due to a lack of retention.

DISCUSSION

To investigate the involvement of RNA2-encoded proteins in the specificity of GFLV transmission by *X. index,* we developed chimeric RNA2 constructs by swapping genes between GFLV and ArMV. Subsequently, we tested the *X. index*-mediated transmissibility of recombinant viruses derived from transcripts of GFLV RNA1 and chimeric RNA2. Transmission of recombinant viruses $2A_UBC$, $2AB_{U9}C$, and $2A_UB_{U9}C$ reached 100, 87, and 89%, respectively, compared to 100 and 95% for natural wild-type GFLV-F13 and synthetic wild-type 2ABC, respectively. Thus, the GFLV determinants for the specificity of transmission are located within the 513 C-terminal residues of polyprotein P2, i.e., the 9 C-terminal $2B^{MP}$ and 504 $2C^{CP}$ amino acids. These findings indicate that neither protein 2A nor protein $2B^{MP}$, except for its 9 C-terminal residues, is responsible for the specific spread of GFLV by *X. index.* This is the first molecular scrutiny into the specificity of a nepovirus transmission.

Our findings suggest that the coat protein (CP) probably provides the basic determinants for the specificity of GFLV transmission by *X. index.* Although our results rule out the involvement of proteins 2A and $2B^{MP}$, except for its 9 C-terminal amino acids, in the specificity of the transmission process, we could not directly demonstrate the involvement of protein $2C^{CP}$ since recombinant viruses $2ABC_U$ and $2AB_{U9}C_U$ are not infectious *in planta* (Belin *et al.,* 1999). Therefore, we could not test their transmissibility by *X. index.* To overcome this limitation, an alternative could be the use of an artificial virus acquisition setting similar to the feeding on membranes, a common approach for aphid transmission experiments. Unfortunately, such an artificial virus acquisition setting is not available for nematode-borne viruses (Brown *et al.,* 1995; Wyss, 2000). Despite the lack of direct molecular evidence to support the implication of protein $2C^{CP}$ in the transmission process, it is very likely involved because serological properties of virus particles correlate with transmissibility (Harrison and Murant, 1977; Harrison *et al.,* 1974a). Also, monolayers of GFLV particles are associated with the surface of the cuticle lining the lumen of the food canal from the most anterior part of the odontophore of *X. index* to the posterior end of the esophageal basal bulb (Brown *et al.,* 1995; Martelli and Taylor, 1990; Wyss, 2000).

It has been previously suggested that a protein other than the CP may be involved in the interaction of nepoviruses with their nematode vectors (Blok *et al.,* 1992; Mayo *et al.,* 1994; Mayo and Robinson, 1996). In particular, protein $2B^{MP}$ has been tentatively proposed to determine the specificity of the transmission process. Based on stretches of identical or nearly identical amino acid sequences between GFLV and *Tomato ringspot virus* (ToRSV) in the $2B^{MP}$ domain, it has been speculated that these viruses may attach to structures specific to *Xiphinema* vectors since GFLV and ToRSV are transmitted by *Xiphinema* species. A similar situation applies to RpRSV and TBRV, which are transmitted by *Longidorus* vectors. Interestingly, the stretches of identical sequences are not common to all four nepoviruses. Thus, it has been proposed that protein $2B^{MP}$ may bind to structures in the nematodes that differ between the two genera. Also, the presence of tubular structures in the food canal of *Longidorus apulus* that fed on plants infected with *Artichoke Italian latent virus* (Mayo *et al.,* 1994) suggested that protein $2B^{MP}$ may play a role in the specificity of *Nepovirus* spread. Interestingly, our findings rule out the involvement of protein $2B^{MP}$, except for possibly its 9 C-terminal residues, in the specificity of transmission. Nonetheless, protein $2B^{MP}$ may act as a nonspecific determinant.

Our study confirmed the specific association between GFLV and *X. index* because synthetic wild-type virus 2ABC, unlike ArMV, was readily detected in *X. index* by IC-RT-PCR (Fig. 5). This specificity of interaction was previously highlighted by the presence of GFLV particles in *X. index* but not in *X. diversicaudatum* (Harrison *et al.,* 1974b). Therefore, it is likely, at least for the *Xiphinema*-transmitted nepoviruses, that vector specificity is related to the specificity of retention of virus particles in the vector. Interestingly, ArMV particles were found in *L. elongatus,* an ArMV-nonvector nematode species, after nematode feeding on ArMV-infected plants (for review see Brown *et al.,* 1995). Thus, it seems that vector specificity is rather related to the specificity of dissociation of virus particles in *Longidorus* vectors. Interestingly, the

CP of the *Longidorus*-transmitted TBRV possesses a proteinase-sensitive structure of 9 amino acids at its C-terminal end (Demangeat *et al.,* 1992). These 9 residues may constitute an "arm-like" structure which could be involved in the release of TBRV particles from nematode retention sites (Mayo *et al.,* 1994). In the case of GFLV, the CP is very stable and a degradation product released upon proteinase activity has never been observed (G. Demangeat, unpublished observations), reinforcing the idea that retention likely determines the specificity of GFLV transmission.

The mechanism by which GFLV is attached to retention sites and subsequently dissociated from them is largely unknown although substantial progress has been made in understanding the feeding behavior of *X. index* (Wyss, 2000). Also, it is unclear whether GFLV particles are directly linked to the odontophore of *X. index* or need another viral protein. Do the 9 C-terminal residues of GFLV protein $2B^{MP}$ play a role in bridging virus particles to retention sites in the odontophore of *X. index* and act as helper (Mayo *et al.,* 1994; Mayo and Robinson, 1996)? Defining the degree of involvement of the different residues composing the 9 amino-acid C-terminal structure of protein $2B^{MP}$ appears to be particularly complex because they are critical for systemic spread *in planta* and for polyprotein P2 processing (Belin *et al.,* 1999). Further, we cannot exclude the possibility that a GFLV-encoded protein, in addition to the 9 C-terminal residues of $2B^{MP}$, could act as a nonspecific helper in the transmission process. Such a helper factor could be encoded by either RNA1 or RNA2, including proteins 2A and $2B^{MP}$. Additional efforts are needed to address the role of GFLV-encoded proteins as a nonspecific determinant in the *X. index*-mediated transmission.

X. index-transmitted recombinant viruses were stable because their progeny showed very minor modifications in the polyprotein P2 sequence. Only single amino acid changes were observed in single but not in all recipient plants tested. Since residues involved in specific GFLV–*X. index* interactions are expected to be present in the progeny of recombinant viruses in all recipient plants, the changes observed probably reflect permissible variability in the viral genome.

The synthetic wild-type virus 2ABC corresponding to transcripts Tr1 and Tr2ABC is devoid of satellite RNA. Thus, it differs from the natural wild-type GFLV-F13, which carries a satellite RNA (Fuchs *et al.,* 1989). The similar transmission rate of GFLV-F13 and synthetic virus 2ABC (Table 1) definitely excludes the requirement of the satellite-encoded 37-kDa protein for transmission.

Petunia was used as virus source plant. Interestingly, no galls were observed during virus acquisition by *X. index,* although *Xiphinema* spp. are known to induce root-tip galls on *Petunia hybrida* (Roberts and Brown, 1980). The absence of galls may be attributable to our population of *X. index.* Indeed, differences in host status among populations of *X. index* have been reported (Coiro and Serino, 1991). Nevertheless, *X. index* exposed to petunia acquired and transmitted wild-type GFLV and recombinant viruses with the same efficiency as those exposed to grapevines, indicating that the development of galls is not a prerequisite for an efficient virus transmission. Therefore, virus spread by *X. index* can be tested using a herbaceous plant that does not produce root galls.

In addition to considerations on the specificity of transmission, our results clearly demonstrate that protein 2A, which is involved in RNA2 replication (Gaire *et al.,* 1999), is active in a heterologous context. Recombinant viruses $2A_UBC$ and $2A_UB_{U9}C$ with protein 2A from ArMV RNA2-U were infectious *in planta.* Thus, protein 2A is functional in a system where the RNA1-encoded RNA-dependent RNA polymerase and the RNA2 noncoding regions originate from GFLV.

In summary, we have shown that the determinants for specific spread of GFLV by *X. index* are located within the 513 C-terminal residues of polyprotein P2. Because of the critical requirement of the 9 C-terminal residues of protein $2B^{MP}$ for proteolytic processing at the R/G site and for systemic spread of the virus (Belin *et al.,* 1999), point mutations are needed to determine whether they play a role in transmission or whether protein $2C^{CP}$ accounts by itself for vector specificity. Such mutational experiments are in progress in our laboratory. If $2C^{CP}$ is the sole GFLV determinant of vector specificity, nematode transmissible viruses would be of two categories. The first category would comprise tobravirus species for which the CP and a nonstructural protein (protein 2b) determine vector specificity (MacFarlane, 1999; Vassilakos *et al.,* 2001; Visser and Bol, 1999). The second category would comprise nepovirus species, which require only the CP. If proven, this situation would be similar to that of aphid transmissible viruses for which potyvirus species use the CP and a helper protein for their transmission (Pirone and Blanc, 1996), whereas cucumovirus species rely solely on the CP (Chen and Francki, 1990).

MATERIALS AND METHODS

Virus strains

GFLV strain F13 (GFLV-F13) and ArMV strain S (ArMV-S) were isolated from naturally infected grapevines and transferred by mechanical inoculation to *C. quinoa,* a systemic herbaceous host for both viruses. GFLV-F13 and ArMV-S were used in this study for cDNA synthesis and nematode transmission assays.

Development of full-length and chimeric RNA2

Plasmid pVec, a pUC-derived vector, was engineered to contain the 5′ and 3′ noncoding regions of GFLV RNA2 under the control of promoter T7. It was used to produce

pVec2ABC carrying the full-length GFLV RNA2 in which *Nhe*I and *Nae*I restriction sites were added immediately downstream of the $2B^{MP}/2C^{CP}$ cleavage site without modifying the amino acid sequence (Belin *et al.,* 1999). Plasmid pVec2ABC was used to develop chimeric RNA2 by exchanging the 2A, $2B^{MP}$, and/or $2C^{CP}$ genes between GFLV-F13 and ArMV-S (Fig. 1B).

The development of three of the chimeric constructs which were used in this study, i.e., $2AB_{U9}C$, $2ABC_U$, and $2AB_{U9}C_U$, was described by Belin *et al.* (1999). Briefly, the fragment encoding $2B^{MP}$, except for its 9 C-terminal residues, originates from ArMV RNA2-U in $pVec2AB_{U9}C$ (Fig. 1B). The complete $2C^{CP}$ gene from ArMV RNA2-U was cloned into pVec2ABC to yield $pVec2ABC_U$ and into $pVec2AB_{U9}C$ to yield $pVec2AB_{U9}C_U$ (Belin *et al.,* 1999).

Two other chimeric constructs were subsequently developed. The ArMV RNA2-U was amplified by PCR using plasmid p60 (Loudes *et al.,* 1995) as template and primer 5EcoS (5′-TTTAAAAAGCTTGATATCAACTATGGGCAAGTTTTATTATAGTAA-3′), which corresponds to the 5′-terminal sequence of the 2A gene with an additional *Eco*RV site (underlined), and primer 3EcoR (5′-TCTAGAGATATCTACGTATCATTAAACTTTAAAGCACGTCC-3′), which corresponds to the 3′-terminal sequence of the $2C^{CP}$ gene with an additional *Eco*RV site (underlined). The PCR-amplified DNA product was cloned into *Eco*RV-digested pVec2ABC to give $pVec2A_UB_UC_U$. The 2A gene of $pVec2A_UB_UC_U$ was then excised by *Age*I and *Alw*NI (present in the vector, upstream of the T7 promoter) and cloned into $pVec2AB_{U9}C$ to give $pVec2A_UB_{U9}C$ (Fig. 1B).

The *Age*I–*Bam*HI fragment (nt 1020–2300) of pVec2ABC was cloned into $pVec2A_UB_{U9}C$ to give $pVec2A_UBC$ (Fig. 1B).

In vitro transcription of chimeric RNA2

Capped transcripts of GFLV RNA1 (Tr1) and RNA2 or chimeric RNA2 (Tr2) were synthesized *in vitro* from cloned full-length cDNAs using the RiboMAX system (Promega) according to the manufacturer's instructions. Plasmid pMV13 (Viry *et al.,* 1993) linearized with *Bgl*II served as template to produce the infectious transcript of GFLV RNA1. Transcripts corresponding to synthetic wild-type (Tr2ABC) or chimeric ($Tr2A_UBC$, $Tr2AB_{U9}C$, $Tr2A_UB_{U9}C$, $Tr2ABC_U$, and $Tr2AB_{U9}C_U$) RNA2 were synthesized from the corresponding plasmids previously linearized with *Sal*I. Transcript size and integrity were checked by agarose–formaldehyde gel electrophoresis (Sambrook *et al.,* 1989) prior to inoculation experiments.

In vitro translation of chimeric RNA2 transcripts and polyprotein P2 processing

RNA2-encoded proteins were translated using the rabbit reticulocyte lysate translation system TNT (Promega) according to the manufacturer's instructions. Maturation of the polyprotein P2 encoded by synthetic wild-type and chimeric RNA2 transcripts was carried out by the GFLV RNA1-encoded $1D^{Pro}$ proteinase, as previously described by Margis *et al.* (1991).

Inoculation of *C. quinoa* and petunia plants

C. quinoa plants were mechanically inoculated according to Viry *et al.* (1993) with either purified wild-type virus (GFLV-F13 or ArMV-S) or transcripts: Tr1 and Tr2ABC corresponding to construct 2ABC, Tr1 and $Tr2A_UBC$ corresponding to construct $2A_UBC$, Tr1 and $Tr2AB_{U9}C$ corresponding to construct $2AB_{U9}C$, Tr1 and $Tr2A_UB_{U9}C$ corresponding to construct $2A_UB_{U9}C$, Tr1 and $Tr2ABC_U$ corresponding to construct $2ABC_U$, or Tr1 and $Tr2AB_{U9}C_U$ corresponding to construct $2AB_{U9}C_U$. The successful establishment of systemic infection in *C. quinoa* was analyzed by monitoring symptom development and by detecting the $2B^{MP}$ protein in total protein extracts from noninoculated apical leaves in Western blotting using an antiserum to the $2B^{MP}$ protein according to Ritzenthaler *et al.* (1995a). This antiserum equally detects the GFLV and ArMV $2B^{MP}$ proteins (Belin *et al.,* 1999).

Petunia (*P. hybrida* spp.) plants were mechanically inoculated at the 4- to 6-leaf stage with wild-type or recombinant viruses which were purified from infected *C. quinoa* plants as described by Pinck *et al.* (1988).

In vitro inoculation of grapevine plants by heterologous grafting

Grapevine variety *Vitis vinifera* × *V. rupestris,* ARG1 was inoculated *in vitro* by heterologous grafting (Bass and Vuittenez, 1979). Briefly, healthy grapevine cuttings were used as scions and stems of virus-infected *C. quinoa* were used as rootstocks. Alternatively, the grapevine rootstock varieties *V. berlandieri* × *V. riparia,* Kober 5BB and *V. berlandieri* × *V. rupestris,* 110R were used. Plant material was collected in the greenhouse and sterilized in a laminar flow hood by soaking in a 70 g/liter sodium hypochlorite solution supplemented with 1% Tween 20 for 20 min followed by three washes in sterile water for 10 min. The extremities of the plant fragments damaged by the sodium hypochlorite solution were removed. Stems of infected *C. quinoa* plants collected 2 weeks postinoculation were fragmented in 2- to 3-cm pieces. Wedge-shaped, one-bud grapevine cuttings and V-shaped *C. quinoa* stem fragments with equivalent sections were then grafted and maintained in glass tubes by a bunched paper bridge, so that only *C. quinoa* stem fragments were in contact with the liquid culture medium [basal MS; Murashige and Skoog (1962); with 2× vitamins, 75 g/liter sucrose, and 0.05 mg/liter α-naphtalenacetic acid]. After 3 weeks of contact at 25°C with a photoperiod of 16 h of light/8 h of dark, rooted grapevine plantlets were separated from *C. quinoa* stems and transferred onto a solidified amended MS medium [basal

MS; Murashige and Skoog (1962); with 1× vitamins, 30 g/liter sucrose, 2 mg/liter glutamine, and 7 g/liter agar] supplemented with 0.15 mg/liter of indolebutyric acid. After 1 month of culture, grapevine plants were progressively acclimatized in sandy soil in the greenhouse and kept for 1 month before nematode transmission experiments. The success of heterologous grafting at transmitting viruses was assessed in grapevine roots by ELISA with GFLV-specific γ-globulins (Walter and Etienne, 1987).

Nematode transmission assay

The transmissibility of wild-type GFLV-F13 and ArMV-S, synthetic wild-type 2ABC, and recombinant isolates $2A_UBC$, $2AB_{U9}C$, and $2A_UB_{U9}C$ was assessed under greenhouse conditions using aviruliferous *X. index* from a 2-year-old rearing on fig plants (*Ficus carica*). Rearing of aviruliferous nematodes was performed in 20-liter containers in a temperature controlled greenhouse (20 ± 2°C). Nematodes were extracted from soil samples using the sieving method described by Flegg (1967) and subsequently counted with a binocular.

A two-step transmission procedure was used. The first step consisted of virus acquisition from a virus source plant. It was carried out in 0.5-liter plastic pots by allowing 300 nematodes to feed on a single virus source plant for a 6-week period. Infected grapevine and petunia plants were used as virus source plants. The grapevine rootstock ARG1 was used as recipient for its susceptibility to *X. index.* The presence of the virus was verified in young roots of source plants by ELISA using γ-globulins (Walter and Etienne, 1987) prior to virus acquisition. At the end of the acquisition step, formation of galls was checked on rootlets of source plants as indicative of nematode feeding. The second step subsequently consisted of virus transmission to a healthy plant. The virus source plant was carefully removed from the pots and replaced by a recipient plant. Nematode feeding occurred for an additional 6-week period. After transmission, roots of the recipient grapevines were checked for gall formation and abundantly washed with water to eliminate adhering soil or nematodes. Virus transmissibility was evaluated in rootlets of recipient plants by ELISA and IC-RT-PCR. The recipient grapevines were subsequently transplanted to a nematode-free soil and maintained in the greenhouse for 5–8 months. ELISA was then performed on major roots of recipient plants to ensure that virus infection in rootlets was not due to contamination by remaining nematodes. Further, recipient grapevines were exposed to cold storage to induce dormancy. After a 1- to 2-month dormancy period, young leaves from newly developed shoots were tested for GFLV by ELISA to confirm infection and subsequently systemic virus spread.

Virus detection by IC-RT-PCR

Nematode extracts were prepared by grinding 30–100 nematodes in a microfuge tube with a plastic pestle in 200 μl of extraction buffer (Walter and Etienne, 1987). Plant rootlets or leaves were ground in the same grinding buffer at a 1/5 ratio (w/v). For IC-RT-PCR, 0.5-ml reaction tubes were coated with specific GFLV-F13 or ArMV-S γ-globulins in coating buffer (0.05 M sodium carbonate, pH 9.6) for 3 h at 37°C and washed three times with PBST [0.15 M NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 0.05% (v/v) Tween 20]. The coated tubes were incubated with 200 μl of nematode extract or plant sap for 3 h at 37°C and washed three times with PBST. Virus particles were disrupted and viral RNA was released by heating for 10 min at 65°C in the presence of 10 μl of 0.1% of Triton X-100. After cooling on ice, reverse transcription was performed with Expand reverse transcriptase (Boehringer) according to the manufacturer's instructions. Amplification of cDNAs was carried out with either primers ArSens (5′-GAGTTTGAGGCAGCAAAGAG-3′) and ArRev (5′-ACAACACACTGTCGCCAC-3′) or primers GfSens (5′-TTGTGCGCCCAGATCTCTCTTTA-3′) and GfRev (5′-ACACATATATACACTTGGGTCTTT-3′) to give a 1177-bp ArMV-specific fragment or a 556-bp GFLV-specific fragment, respectively.

Characterization of recombinant RNA2 progeny

The progeny of chimeric RNA2 were characterized by IC-RT-PCR followed by sequencing. Roots of recipient plants were used for immunocapture and cDNA synthesis in two successive 25-cycle PCRs with the high-fidelity PCR master kit (Boehringer). Amplified fragments were purified using the GeneClean kit (Bio101) and sequenced using the AmpliTAQ FS polymerase with rhodamine dye terminator and an Applied Biosystems 373 sequencer (Perkin–Elmer).

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REFERENCES

Ayllon, M., Rubio, L., Moya, A., Guerri, J., and Moreno, P. (1999). The haplotype distribution of two genes of citrus tristeza virus is altered after host change or aphid transmission. *Virology* **255,** 32–39.

Bass, P., and Vuittenez, A. (1979). Méthodes d'inoculation virale par hétérogreffage entre plantes hôtes herbacées et ligneuses en culture aseptique *in vitro.* Application à la transmission de souches définies de népovirus de *C. quinoa* à la vigne *Vitis* ssp. *Ann. Phytopathol.* **11,** 565–567.

Belin, C., Schmitt, C., Gaire, F., Walter, B., Demangeat, G., and Pinck, L. (1999). The nine C-terminal residues of the grapevine fanleaf nepovirus movement protein are critical for systemic virus spread. *J. Gen. Virol.* **80,** 1347–1356.

Blok, V. C., Wardell, J., Jolly, C., Manoukian, A., Robinson, D. J., Edwards,

M. L., and Mayo, M. A. (1992). The nucleotide sequence of RNA2 of raspberry ringspot nepovirus. *J. Gen. Virol.* **73,** 2189–2194.

Brown, D. J. F., Robertson, W. M., and Trudgill, D. L. (1995). Transmission of viruses by plant nematodes. *Annu. Rev. Phytopathol.* **33,** 223–249.

Chen, B., and Francki, R. I. B. (1990). Cucumovirus transmission by the aphid *Myzus persicae* is determined solely by the viral coat protein. *J. Gen. Virol.* **71,** 939–944.

Coiro, M. I., and Serino, M. (1991). Possible herbaceous hosts for *Xiphinema index* in virus transmission experiment. *Nematol. Medit.* **19,** 251–252.

Demangeat, G., Hemmer, O., Reinbolt, J., Mayo, M., and Fritsch, C. (1992). Virus-specific proteins in cells infected with tomato black ring nepovirus: Evidence for proteolytic processing *in vivo*. *J. Gen. Virol.* **73,** 1609–1614.

Flegg, J. J. M. (1967). Extraction of *Xiphinema* and *Longidorus* species from soil by a modification of Cobb's decanting and sieving technique. *Ann. Appl. Biol.* **60,** 420–437.

Fuchs, M., Pinck, M., Serghini, M. A., Ravelonandro, M., Walter, B., and Pinck, L. (1989). The nucleotide sequence of satellite RNA in grapevine fanleaf virus, strain F13. *J. Gen. Virol.* **70,** 955–962.

Gaire, F., Schmitt, C., Stussi-Garaud, C., Pinck, L., and Ritzenthaler, C. (1999). Protein 2A of grapevine fanleaf nepovirus is implicated in RNA2 replication and colocalizes to the replication site. *Virology* **264,** 25–36.

Harrison, B. D., and Murant, A. F. (1977). Nematode transmissibility of pseudorecombinant isolates of tomato black ring virus. *Ann. Appl. Biol.* **86,** 209–212.

Harrison, B. D., Murant, A. F., Mayo, M. A., and Roberts, I. M. (1974a). Distribution of determinants for symptom production, host range and nematode transmissibility between the two RNA components of raspberry ringspot virus. *J. Gen. Virol.* **22,** 233–247.

Harrison, B. D., Robertson, W. M., and Taylor, C. E. (1974b). Specificity of retention and transmission of viruses by nematodes. *J. Nematol.* **6,** 155–164.

Loudes, A.-M., Ritzenthaler, C., Pinck, M., Serghini, M. A., and Pinck, L. (1995). The 119 kDa and 124 kDa polyproteins of Arabis mosaic nepovirus (isolate S) are encoded by two distinct RNA2 species. *J. Gen. Virol.* **76,** 899–906.

MacFarlane, S. A. (1999). Molecular biology of the tobraviruses. *J. Gen. Virol.* **80,** 2799–2807.

Margis, R., Ritzenthaler, C., Reinbolt, J., Pinck, M., and Pinck, L. (1993). Genome organization of grapevine fanleaf nepovirus RNA2 deduced from the 122K polyprotein P2 *in vitro* cleavage products. *J. Gen. Virol.* **74,** 1919–1926.

Margis, R., Viry, M., Pinck, M., and Pinck, L. (1991). Cloning and *in vitro* characterization of the grapevine fanleaf virus proteinase cistron. *Virology* **185,** 779–787.

Martelli, G. P., and Taylor, C. E. (1990). Distribution of viruses and their nematode vectors. *In* "Advances in Disease Vector Research" (K. Harris, Ed.), Vol. 6, pp. 151–189. Springer-Verlag, New York.

Mayo, M. A., Robertson, W. M., Legorburu, F. J., and Brierley, K. M. (1994). Molecular approaches to an understanding of the transmission of plant viruses by nematodes. *In* "Advances in Molecular Plant Nematology" (F. Lamberti, C. D., Giorgi, and D. Bird, Eds.), pp. 277–293. Plenum, New York.

Mayo, M. A., and Robinson, D. J. (1996). Nepoviruses: Molecular biology and replication. *In* "The Plant Viruses, Vol 5, Polyhedral Virions and Bipartite RNA" (B. D. Harrison and A. F. Murant, Eds.), pp. 139–185. Plenum, New York.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue. *Physiol. Plant.* **15,** 473–497.

Pearson, R. C., and Goheen, A. (1991). Fanleaf degeneration. *In* "Compendium of Grape Diseases" (T. A. P. Society, Ed.), pp. 48–49. APS Press, St. Paul, MN.

Pinck, L., Fuchs, M., Pinck, M., Ravelonandro, M., and Walter, B. (1988). A satellite RNA in grapevine fanleaf virus strain F13. *J. Gen. Virol.* **69,** 233–239.

Pirone, T. P., and Blanc, S. (1996). Helper dependent vector transmission of plant viruses. *Annu. Rev. Phytopathol.* **34,** 227–247.

Ritzenthaler, C., Pinck, M., and Pinck, L. (1995a). Grapevine fanleaf nepovirus P38 putative movement protein is not transiently expressed and is a stable final maturation product *in vivo*. *J. Gen. Virol.* **76,** 907–915.

Ritzenthaler, C., Schmit, A.-C., Michler, P., Stussi-Garaud, C., and Pinck, L. (1995b). Grapevine fanleaf nepovirus P38 putative movement protein is located on tubules *in vivo*. *Mol. Plant Microbe Interact.* **8,** 379–387.

Ritzenthaler, C., Viry, M., Pinck, M., Margis, R., Fuchs, M., and Pinck, L. (1991). Complete nucleotide sequence and genetic organization of grapevine fanleaf nepovirus RNA1. *J. Gen. Virol.* **72,** 2357–2365.

Roberts, I. M., and Brown, D. J. F. (1980). Detection of six nepoviruses in their nematode vectors by immunosorbent electron microscopy. *Ann. Appl. Biol.* **96,** 187–192.

Roossinck, M. J. (1997). Mechanisms of plant virus evolution. *Annu. Rev. Phytopathol.* **35,** 191–209.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Serghini, M., Fuchs, M., Pinck, M., Reinbolt, J., Walter, B., and Pinck, L. (1990). RNA2 of grapevine fanleaf virus: Sequence analysis and coat protein cistron location. *J. Gen. Virol.* **71,** 1433–1441.

Vassilakos, N., Vellios, E. K., Brown, E. C., Brown, D. J., and MacFarlane, S. A. (2001). Tobravirus 2b protein acts *in trans* to facilitate transmission by nematodes. *Virology* **279,** 478–487.

Viry, M., Serghini, M. A., Hans, F., Ritzenthaler, C., Pinck, M., and Pinck, L. (1993). Biologically active transcripts from cloned cDNA of genomic grapevine fanleaf nepovirus RNAs. *J. Gen. Virol.* **74,** 169–174.

Visser, P., and Bol, J. (1999). Nonstructural proteins of tobacco rattle virus which have a role in nematode-transmission: Expression pattern and interaction with viral coat protein. *J. Gen. Virol.* **80,** 3273–3280.

Walter, B., and Etienne, L. (1987). Detection of the grapevine fanleaf viruses away from the period of vegetation. *J. Phytopathol.* **120,** 355–364.

Wyss, U. (2000). *Xiphinema index,* maintenance and feeding in monoxenic cultures. *In* "Maintenance of Human, Animal and Plant Pathogen Vectors" (K. Maramorosch and F. Mahmood, Eds.), pp. 251–281. Science Res. Assoc., Chicago.